Two cellulolytic Clostridium species: Clostridium cellulosi sp. nov. and Clostridium cellulosi fermentans sp. nov.

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Two cellulolytic clostridia, one thermophilic and the other mesophilic, were isolated and characterized. Cells of the thermophile are gram-negative rods that are motile with lophotrichous flagella and spherical terminal endospores which swell the cells. The optimum growth temperature is 55°C to 60°C, with a range of 40 to 65°C. The deoxyribonucleic acid composition is 35 mol% G+C. The name Clostridium cellulosi sp. nov. is proposed. The type strain is AS 1.1777. Cells of the mesophile are gram-negative and motile with peritrichous flagella and terminal or spherical spores which swell the cells. The deoxyribonucleic acid composition is 34 mol% G+C. The name Clostridium cellulosi fermentans sp. nov. is proposed. The type strain is AS 1.1775. Both C. cellulosi AS 1.1777 and C. cellulosi fermentans AS 1.1775 are deposited in the China Committee for Culture Collection of Microorganisms, Institute of Microbiology, Academia Sinica, Beijing, People’s Republic of China.

MATERIALS AND METHODS

Media. CM3 medium (14) was used for enrichment and isolation of the thermophile, except that cellulose MN300 was replaced by a cellulose suspension or filter paper strips. A cellulose suspension with a final concentration of 9.0 g per liter and 40 g of agar per liter were used for solid medium. The cellulose suspension consisted of 3% (wt/vol) Whatman paper in each tube. The pH was adjusted to 7.3 to 7.5 with 4 N NaOH.

The solid medium used for isolation of the mesophile consisted of (NH₄)₂SO₄ (1.2 g), yeast extract (2 g), KH₂PO₄ (1.5 g), K₂HPO₄ · 3H₂O (2.0 g), MgCl₂ · 6H₂O (1.0 g), CaCl₂ (0.15 g), FeSO₄ solution (0.1%, wt/vol) (1.25 ml), vitamin solution (0.4 ml), l-cysteine (0.5 g), Na₃P · 9H₂O (0.5 g), resazurin solution (0.2%, wt/vol) (1.0 ml), a 3% (wt/vol) cellulose suspension (300 ml), agar (20 g), and distilled water made up to 1 liter. The pH was adjusted to 7.3 to 7.5 with 4 N NaOH.

The solid medium was dispensed in tubes in 5-ml volumes, and the liquid medium was dispensed in 10-ml volumes. All of the media were autoclaved at 121°C for 15 min.

Anaerobic culture methods. The anaerobic techniques of Hungate (4), as modified by Bryant (1), were used throughout the study. Anaerobic test tubes (16 by 160 mm) sealed with butyl rubber stoppers were used for enrichment and isolation. Oxygen-free nitrogen was used as the atmosphere. Incubation was without shaking at 60°C for the thermophile and 40°C for the mesophile.

Isolation procedures. For isolation of the thermophile, enrichment cultures were inoculated with approximately 2-g samples of cow manure compost into 10 ml of preduced broth medium supplemented with filter paper strips and incubated at 60°C. After the filter paper had decayed, the culture was heat shocked at 90°C for 10 min and 0.5 ml of the 10-ml culture was subcultured into 10 ml of fresh broth medium. This procedure was repeated three times. The final culture was then serially diluted and heat shocked at 90°C for 10 min before the roll tube cultures were made. After 5 days of incubation, single cellulolytic colonies were picked, diluted, heat shocked, and subcultured again in roll tubes. We repeated this procedure five times. Culture purity also was assessed by microscopic examination and examination of roll tubes for noncellulolytic colonies.

Isolation of the mesophile was similar, except that the inoculum was a soil sample from a dairy farm and the temperatures of incubation and heat shock were 40 and 80°C, respectively.

Biochemical reactions. Biochemical characteristics were studied by the methods of Holdeman et al. (3).

Temperature and pH studies. The optimum temperature and pH for growth were determined in PY-cellobiose medium (3) with 0.2 ml of a 48-h-old culture grown in a similar medium. Cultures used to determine the optimum growth pH were incubated at 60°C for the thermophilic isolate and 40°C for the mesophilic isolate.

DNA base composition. Deoxyribonucleic acid (DNA) was extracted and purified by the method of Marmur (8). Moles percent G + C of DNA was determined by thermal denaturation (7) with a Perkin-Elmer Lambda 7 spectrophotometer. 

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FIG. 1. Micrograph of *C. cellulosi* cells stained with safranine. Bar, 10 µm.

Equipped with a temperature program controller. A culture of *Escherichia coli* K-12 was used as a control.

**Electron microscopy.** For electron microscopy, an overnight culture grown in cellubiose broth was harvested by centrifugation at 3,000 × g for 10 min. The pellet was suspended in distilled water and centrifuged as before. This washing procedure was repeated twice. A drop of the suspension of washed cells was placed on a Formvar-coated copper grid, dried at 40°C, and then shadowed with chromium at an angle of 18 to 22°. The cells were examined with a JEOL JEM-100 cx transmission electron microscope.

**Fermentation end product analyses.** The culture headspace gases were analyzed on an SC-3A gas chromatograph with a stainless steel column (2 m by 3 mm [inside diameter]) packed with TDX-01 (60/80 mesh).

Alcohols and volatile and methylated nonvolatile acids were measured on a Shimadzu GC9A gas chromatograph equipped with a flame ionization detector, and the stainless steel column (2 m by 3 mm [inside diameter]) was filled with GDX-401 (60/80 mesh).

**RESULTS AND DISCUSSION**

The isolation procedures yielded pure cultures of two cellulolytic, anaerobic bacteria. As strictly anaerobic and non-sulfate-reducing spore formers, the two bacteria should be placed in the genus *Clostridium* (2). Both are significantly different from previously described cellulolytic clostridia. Therefore, we propose the following new species names: *Clostridium cellulosi* for the thermophilic isolate and *C. cellulofermentans* for the mesophilic isolate.

*Clostridium cellulosi* sp. nov. (*cellulosi*, L. gen. n. of cellulose). Cells are gram-negative, straight or slightly curved rods which usually are 0.3 to 0.6 µm wide by 2.0 to 15.0 µm long. Although most young cells are 2.0 to 4.0 µm long, longer ones are common and cells with spores are frequently longer than vegetative cells (Fig. 1). Cells occur singly, in pairs, or in chains and are motile, with lophotrichous flagella (Fig. 2). Endospores are terminal and spherical and swell the cells. Cultures survive 100°C for 20 min. Heat shock favors germination of the cultures.

In cellulose agar medium, clear zones showing digestion of cellulose are observed after 48 h of incubation. Tiny colonies appear in the centers of the clear zones. During prolonged incubation, the clear zones and colonies expand. Deep colonies are round with entire margins. Surface colonies are usually watery, spreading, and irregular. Colonies in cellulose agar are white, and filter paper strips in broth do not change color.

The optimum temperature for growth is 55 to 60°C, with a...
FIG. 3. Safranine-stained cells of *C. cellulofermentans*. Bar, 10 μm.

range of 40 to 65°C. No growth is observed at 35 or 70°C. The optimum pH for growth is 7.3 to 7.5, and the pH range is 6.2 to 8.5. Ruminal fluid or added vitamins and tryptic peptone are not necessary for growth. Nutritional requirements are met by 2 g of yeast extract per liter. Only slight growth occurs in PY medium with no fermentable carbohydrate. Strictly anaerobic medium is required. No germination occurs in medium in which traces of dissolved oxygen have been indicated by resazurin turning pink.

Catalase, gelatinase, and indole are not produced. Sulfate and nitrate are not reduced. Acetylmethylcarbinol is produced, and milk is curdled. The other biochemical characteristics are given in Table 2.

The major end products of cellulose fermentation are hydrogen, carbon dioxide, ethanol, and acetic acid. Isolated from cow manure compost.

The DNA composition is 35 mol% G+C. The type strain is *C. cellulosi* AS 1.1777.

**FIG. 4.** Electron micrograph of peritrichous flagella of *C. cellulofermentans*. Bar, 0.8 μm.

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**TABLE 2. Differential characteristics of *C. cellulofermentans* and some other clostridia**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>C. cellulofermentans</em></th>
<th><em>C. cellulolyticum</em></th>
<th><em>C. cellulo- C. populi- C. lento-cellum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Esculin</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>W&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Glycogen</td>
<td>–</td>
<td>–</td>
<td>NR</td>
</tr>
<tr>
<td>Inulin</td>
<td>–</td>
<td>–</td>
<td>NR</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>–</td>
<td>–</td>
<td>NR</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>W&lt;sup&gt;b&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>Melibiose</td>
<td>–</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Raffinose</td>
<td>–</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Salicin</td>
<td>–</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Sorbose</td>
<td>–</td>
<td>–</td>
<td>NR</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Gram staining</strong></td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>Optimum temp (°C)</strong></td>
<td>37–40</td>
<td>32–35</td>
<td>37</td>
</tr>
<tr>
<td><strong>Acetylmethylcarbinol</strong></td>
<td>–</td>
<td>–</td>
<td>NR</td>
</tr>
<tr>
<td><strong>Production</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td><strong>G+C content (mol%)</strong></td>
<td>34</td>
<td>41</td>
<td>26–27</td>
</tr>
</tbody>
</table>

<sup>a</sup> All of the strains ferment cellobiose, cellulose, fructose, and glucose but do not ferment erythritol, glycerol, melezitose, rhamnose, and sorbitol.

<sup>b</sup> NR, Not reported.

<sup>c</sup> W, Weakly fermented.

<sup>d</sup> It has been reported that fimbriae are the source of *C. lentocellum* locomotion (9).

*Clostridium cellulofermentans* sp. nov. (*cellulofermentans*, N.L. adj. *cellulose fermenting*). Cells are gram negative, straight or slightly curved rods which are 0.4 to 0.7 μm wide by 1.5 to 7.0 μm long. They occur singly, in pairs, and occasionally in short or long chains. Spherical or oval terminal endospores cause marked swelling of the cells (Fig. 3). Mature spores are 0.8 to 1.2 μm in diameter. They survive 100°C for 10 min. Cells are motile with peritrichous flagella (Fig. 4).

Surface colonies on cellulose agar medium are white, opaque, circular, and flat or slightly convex, with entire or slightly undulate margins. Clear zones in cellulose agar are usually observed after 48 or 72 h of incubation.

Anaerobic medium is necessary for initiation of growth. The optimum growth temperature is 37 to 40°C, and the temperature range is 20 to 45°C. No growth is produced at 15 or 50°C. The optimum pH is 7.0 to 7.2, with a range of 6.0 to 8.2.

Catalase, gelatinase, acetylmethylcarbinol, and indole are not produced. Sulfate is not reduced. Nitrate is reduced, and milk is curdled. Only slight growth occurs in PY medium with no fermentable carbohydrate. The other biochemical characteristics are given in Table 2.

The major end products of cellulose fermentation are hydrogen, carbon dioxide, ethanol, and acetic acid. The ratio of ethanol to acetic acid is about 1:1.

Isolated from soil of a dairy farm.

DNA composition is 34 mol% G+C.
The type strain of *C. cellulofermentans* is strain AS 1.1775.

Both *C. cellulosi* AS 1.1777 and *C. cellulofermentans* AS 1.1775 are deposited in the China Committee for Culture Collection of Microorganisms.

**Distinguishing characteristics of *C. cellulosi***. Valid descriptions of only four species of thermophilic, cellulolytic clostridia have been published. They are *C. thermocellum* (2), *C. stercorarium* (6), *C. thermolaticum* (11), and *C. thermocopriae* (5). They all differ from *C. cellulosi* in several characteristics.

In *C. thermocellum* cultures, both the colonies and the surrounding area representing cellulose hydrolysis are yellow. This strong pigmentation was not observed in and around colonies of *C. cellulosi*. *C. thermocellum* is nonmotile, but *C. cellulosi* is motile with lophotrichous flagella. *C. cellulosi* ferments many more carbohydrates than does *C. thermocellum* (Table 1). *C. cellulosi* curdles milk, but *C. thermocellum* does not. *C. thermocellum* produces gelatinase, but *C. cellulosi* does not. The G+C content of *C. thermocellum* is 38 to 39 mol%, which is higher than that of *C. cellulosi*.

*C. cellulosi* differs from *C. stercorarium* in the fermentations of eight kinds of carbohydrates (Table 1). The G+C contents of *C. cellulosi* and *C. stercorarium* are 35 and 39 mol%, respectively. *C. cellulosi* is lophotrichous, but *C. stercorarium* is peritrichous. Also, *C. cellulosi* produces acetylmethylcarbinol and curdles milk but *C. stercorarium* does not.

The DNA content of *C. thermolaticum* is 41 to 42 mol%, which is much higher than that of *C. cellulosi*. *C. thermolaticum* is also characterized by production of large amounts of lactate from fermentable sugars. Other differences are listed in Table 1.

*C. thermocopriae* is characterized by production of butyric acid, lactate, and hydrogen sulfide. Some other differences between the two species are given in Table 1.

**Distinguishing characteristics of *C. cellulofermentans***. In *Bergey’s Manual of Systematic Bacteriology* (2), three species of cellulolytic, mesophilic clostridia have been described: *C. cellobioparum*, *C. papyrosolvens*, and *C. polysaccharolyticum*.

*C. cellulofermentans* differs from *C. cellobioparum* by reducing nitrate, curdling milk, and fermenting glycogen, inulin, raffinose, and trehalose but not fermenting arabinose. Also, the DNA base composition of *C. cellobioparum* is 28 mol% G+C, which is much lower than that of *C. cellulofermentans*.

*C. papyrosolvens* has a lower optimum growth temperature of 20 to 30°C and differs from *C. cellulofermentans* in not reducing nitrate, not curdling milk, and not fermenting glycogen, inulin, maltose, mannitol, mannoose, melibiose, raffinose, salicin, sucrose, and trehalose.

The DNA base composition of *C. polysaccharolyticum* is 42 mol% G+C, which is much higher than that of *C. cellulofermentans*. *C. polysaccharolyticum* is also distinct from *C. cellulofermentans* in not reducing nitrate, not curdling milk, and not fermenting fructose, galactose, inulin, lactose, maltose, mannitol, mannoose, melibiose, raffinose, salicin, sucrose, and trehalose.

Recently, valid descriptions of four new species of cellulolytic, mesophilic clostridia have been published. They are *C. cellulosilyticum* (10), *C. celluvorans* (13), *C. populeti* (12), and *C. lentocellum* (9). Table 2 lists the characteristics of the four species that distinguish them from *C. cellulofermentans*.

**REFERENCES**


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